Cyclosporin Protects the Eyelid Skin From Injury After Injection of Doxorubicin

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Purpose. The myotoxic drug doxorubicin can treat blepharospasm and hemifacial spasm permanently when injected directly into the eyelid of patients. One side effect of this treatment is the dose-related occurrence of injury to the skin overlying the injection site. The purpose of this study was to determine if injection of the immunosuppressive agent cyclosporin into rabbit eyelids before doxorubicin treatment could reduce the occurrence of injury to the overlying skin and to determine the effect of cyclosporin pretreatment on doxorubicin-induced muscle fiber loss.

Methods. Anesthetized rabbits received injections of varying doses of cyclosporin 20 minutes before injection of either 0.5, 1, or 2 mg doxorubicin. The rabbits were examined daily, and epithelial changes were recorded as to duration, time of onset, and healing. When the skin was completely healed, the animals were killed and eyelid tissue was prepared for morphometric determination of muscle fiber number. Acute inflammation was quantitatively assessed using an Evans blue assay.

Results. At specified doses, cyclosporin improved the doxorubicin chemomyectomy protocol in three ways. It delayed the onset of skin injury at the higher doses of doxorubicin, and it markedly decreased the duration of skin injury. At some doses, cyclosporin completely prevented the formation of epithelial defects. The combination, however, did not increase muscle loss compared to doxorubicin alone; in fact, it had a slightly myoprotective effect. A dose range for cyclosporin administration was determined that resulted in a quantitative and dose-dependent reduction in inflammation at the injection site.

Conclusions. The injection of cyclosporin into the eyelids before doxorubicin treatment delayed the onset, reduced the duration, and limited the extent of development of eyelid skin injury. Perhaps by limiting cytokine release, cyclosporin decreased the inflammatory reaction compared to that seen with doxorubicin alone. This combination has the potential to improve patient acceptance of doxorubicin chemomyectomy for the treatment of blepharospasm and hemifacial spasm. Invest Ophthalmol Vis Sci. 1995;36:1433-1440.
when used in combination with doxorubicin, have increased muscle loss in the eyelid. However, these combined treatments did not reduce the occurrence or duration of skin injury, and, in fact, some pretreatments such as collagenase increased the size and duration of the resultant skin injury. To reduce the occurrence of skin injury overlying the site of injection of doxorubicin into the eyelids while simultaneously trying to increase the muscle loss over that seen with injection of doxorubicin only, cyclosporin was injected before doxorubicin treatment of the rabbit eyelids.

Cyclosporin is a cyclic polypeptide known to have immunosuppressive effects. In numerous studies, cyclosporin has been shown to increase the toxicity of doxorubicin and related anthracyclines when used for cancer treatment. Cyclosporin treatment resulted in decreased clearance of doxorubicin from cells, as well as an increased accumulation of doxorubicin and a decreased breakdown of doxorubicin to the inactive aglycone forms. Cyclosporin is highly lipid soluble, distributes readily across most membranes, and alters membrane architecture. It inhibits cytokine release, particularly interleukin-2, and thus has the potential to alter the localized acute inflammatory reaction caused by doxorubicin. It seemed a good candidate to use, in combination with doxorubicin, to maximize muscle injury while providing some protection to the skin.

METHODS

New Zealand white rabbits were obtained from Birchwood Valley Farm (Red Wing, MN) and housed with Research Animal Resources at the University of Minnesota. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rabbits were anesthetized with an intramuscular injection of ketamine:xylazine, 1:1, (10 mg/kg and 2 mg/kg, respectively). The lower eyelids of each rabbit were injected with cyclosporin (Sandimmune, Sandoz Ltd., Basel, Switzerland) at a dose of either 10 mg, 5 mg, 2 mg, 1 mg, or 0.5 mg, with a minimum of four eyelids treated at each dose, with the exception of 10 mg (two lids) and 5 mg (three lids). The cyclosporin was diluted in sterile isotonic saline, and all injection volumes were held at 0.4 mm per eyelid. Twenty minutes after injection of cyclosporin, the lower eyelids were injected with either 2 mg, 1 mg, or 0.5 mg doxorubicin (Adriamycin, Adria Labs, Columbus, OH) diluted in sterile isotonic saline, with all injection volumes at 0.4 ml. Between four and six eyelids were treated for each of the dosages of cyclosporin and doxorubicin. An additional four lower eyelids at each dose were injected with 2 mg, 1 mg or 0.5 mg doxorubicin only. An additional four lower eyelids were injected with 5-mg, 1-mg, or 0.5-mg doses of cyclosporin only.

Every day after the initial injections, the eyelids were examined for changes in the skin. Records were kept of the day of onset, duration, and size of the epithelial defects that appeared in the treated eyelids.

When the skin was completely healed, the rabbits were killed by an overdose of barbiturate anesthesia. The treated eyelids were removed and prepared for histologic examination. Samples were frozen in 5-methylbutane that had been chilled to a slurry in liquid nitrogen. The tissue was sectioned at 12 μm and processed for myosin ATPase histochemistry for differentiation of type 1 and type 2 fiber types. Five representative sections through each eyelid cross-section were examined morphometrically. Individual fibers were quantified microscopically with the aid of an image analysis system (Bioquant, R & M Biometrics, Nashville, TN).

To assess whether the cyclosporin altered the inflammatory reaction at the injection site, five eyelids for each of the following parameters were prepared: 1 mg doxorubicin, 1 mg cyclosporin, 2 mg cyclosporin, 1 mg cyclosporin followed 20 minutes later by 1 mg doxorubicin, 2 mg cyclosporin followed 20 minutes later by 1 mg doxorubicin, saline, and no injection. Twenty-four hours after these injections, the animals were assessed for inflammation at the injection site using the Evans blue protocol to quantify vascular permeability. The animals were deeply anesthetized and injected through the ear vein with a solution of Evans blue (50 mg/kg). After 10 minutes of systemic spread, the rabbits were perfused with a constant volume of a solution of 1:1 phosphate-buffered saline—sucrose. Tissue was removed from each eyelid, with care taken not to spread any extraneous Evans blue on the tissue sample. Individual tissue samples were placed in test tubes containing formamide and then in a 57°C water bath overnight. The next day, the formamide solutions were analyzed on a visible light spectrophotometer at 620 nm.

All results were compared for statistical significance using an unpaired, two-tailed test. An F-test indicated that the variances of the control and experimental groups were not significantly different. Statistical tests were run using the Instat biostatistics program (Graphpad, San Diego, CA).

RESULTS

Cyclosporin pretreatment of the eyelid before injection with doxorubicin substantially increased the number of days to the first appearance of an epithelial defect (Fig. 1). The higher doses of cyclosporin, namely 10 mg and 5 mg, followed by doxorubicin appeared to have toxic properties that increased skin...
DAYS TO ONSET OF EPITHELIAL DEFECT

FIGURE 1. Graph of days until onset of an epithelial defect after doxorubicin only or combined cyclosporin and doxorubicin treatment. nd = dose not examined. *Significant difference from the doxorubicin-only animals; + no sores developed at that dose.

injury. These doses were dropped from further study. As the dose of cyclosporin was reduced from 5 mg to 1 mg, the number of days to the onset of epithelial defects increased compared with doxorubicin alone. The most effective dose for delaying onset of skin injury was 1 mg cyclosporin. When the dose of cyclosporin was further reduced, the days to onset of epithelial changes again decreased.

Epithelial defects developed in all eyelids injected with doxorubicin at doses of 2 mg, 1 mg, or 0.5 mg (Fig. 2). When cyclosporin was injected before doxorubicin, for each cyclosporin dose administered, there were always some animals in which skin injury did not develop at all (Fig. 2). Doses of 2, 5, or 10 mg cyclosporin were less effective than lower doses at preventing the development of epithelial defects. When cyclosporin was administered in a dose of 1 mg or 0.5 mg per eyelid, maximal protection of eyelid skin was achieved. After injection of 0.5 mg cyclosporin and 1 mg doxorubicin, epithelial defects did not develop in any of the treated eyelids.

The most dramatic effect of the cyclosporin pre-treatment of the eyelids before doxorubicin injection was a marked decrease in the duration of the epithelial defect on the eyelid skin (Fig. 3). With doxorubicin treatment only, the defect was present for 3 to 5 weeks. Pretreatment with 2 mg cyclosporin before doxorubicin injection reduced the duration of epithelial defects at all concentrations of doxorubicin. It was particularly effective at the lowest concentration of doxorubi-
bacin. Pretreatment with 1 mg cyclosporin again reduced the duration of the epithelial defect at all concentrations of doxorubicin used. The combination of 1 mg cyclosporin and 1 mg or 0.5 mg doxorubicin resulted in the shortest duration of skin changes of any treatment. Pretreatment with 0.5 mg cyclosporin reduced the duration of the epithelial defects compared to doxorubicin alone, but it was less effective than pretreatment with 1 mg cyclosporin.

Microscopic examination of the doxorubicin- and doxorubicin and cyclosporin-treated eyelids showed a clear loss of muscle fibers in the treated eyelids compared to controls (Fig. 4). It appeared, however, that high doses of doxorubicin alone were more effective in killing orbicularis oculi muscle fibers than the combination of doxorubicin and cyclosporin (Fig. 4). The muscle loss was quantified for each of the experimental parameters (Fig. 5). There was a significant decrease in muscle fiber number compared to controls for all experimental parameters. The most effective treatments for muscle loss were either 1 or 2 mg doxorubicin alone, which resulted in a loss of 81% and 88%, respectively. Cyclosporin apparently had a mild muscle-protective effect when injected before doxorubicin treatment, with a 60% to 70% muscle loss seen when the lids were cotreated with both drugs. There was no significant difference between muscle loss seen with 0.5 mg doxorubicin alone and doxorubicin given with any of the cyclosporin injections. Thus, although cyclosporin did not enhance muscle loss when compared to doxorubicin alone, it still significantly decreased the time to onset and duration of skin injury. Cyclosporin alone did not alter the muscle fiber number in the treated eyelids compared to controls (data not shown).

We examined the effect of cyclosporin on the inflammatory reaction that occurs in the eyelid after doxorubicin injection and doxorubicin injected subsequent to cyclosporin injection by using an Evans blue assay for quantitation of vascular permeability (Fig. 6). Injection of doxorubicin alone resulted in a significant increase in vascular permeability in the treated eyelids. Both 1 mg cyclosporin and 2 mg cyclosporin decreased the vascular permeability when injected 20 minutes before doxorubicin injection at the same dose, although this difference was only significant after the injection of 2 mg cyclosporin.

**DISCUSSION**

The injection of cyclosporin into the eyelids of rabbits before doxorubicin injection substantially delayed the onset, decreased the duration, and in some cases even prevented the development of eyelid skin injury. This represents a major improvement of the doxorubicin chemomyectomy protocol for use in the treatment of patients with either blepharospasm or hemifacial spasm. As reported, as the dose of doxorubicin was increased and the number of injections was increased, the likelihood of skin injury was increased. Decreasing the inflammatory reaction at the injection site and decreasing skin injury should increase patient accep-
tance of this treatment because it appears to add to safety without a significant decrease in efficacy. The results reported here reflect the result of a single injection cycle in rabbits. In humans, it is still likely that more than one injection cycle is required, hence, the effect of adding cyclosporin to multiple injection cycles in human eyelids remains to be determined. Because the clinical effect of doxorubicin chemomyectomy may depend not only on chemomyectomy but also on the extent and distribution of subcutaneous scarring within the treated lid, the clinical effect of diminished scar formation is difficult to predict.

The mechanism for initiation of the inflammatory action of doxorubicin at the site of localized injection is probably multifactorial. Doxorubicin toxicity is thought to result from its local production of free-oxygen radicals and the subsequent abnormalities in calcium-transport mechanisms. Signs of muscle injury are seen as early as 5 minutes after doxorubicin injection. The resultant muscle necrosis would, in turn, cause infiltration of lymphocytes and macrophages to the injection site. Doxorubicin also results in enhanced release of a number of cytokines, including interleukin-1 and interleukin-2. This would stimulate immune system activation at the injection site. Thus, it is not surprising that a local injection of doxorubicin results in a pronounced inflammatory reaction at the site of drug application.

Local extravasation of doxorubicin during intravenous administration has long been known to cause tissue destruction, in part because of the persistence of doxorubicin in human skin long after extravasation (McLoon, unpublished observations, 1995). Numerous drugs have been used to treat doxorubicin skin toxicity, but none have been particularly effective. Although the mechanism of action of cyclosporin is not understood, it has been described as having both humoral and cell-mediated effects in immune system suppression. Specifically, cyclosporin inhibits interleukin-2 release and prevents activation of T helper cells and T cytotoxic cells, but it must be present immediately after cell stimulation.

FIGURE 4. Photomicrographs of orbicularis oculi muscle. A, C, and E are pretarsal regions. B, D, and F are preseptal regions. A and B are normal untreated muscles; C and D are treated with 2 mg cyclosporin and 2 mg doxorubicin and show muscle fiber loss; E, F are treated with 2 mg doxorubicin and show extensive muscle fiber loss, with complete fiber loss in the preseptal portion of the muscle.
Muscle Fiber Number
Cyclosporin and Doxorubicin

FIGURE 5. Graph of muscle fiber number in the doxorubicin-treated and the cyclosporin and doxorubicin treated orbicularis oculi muscles. All treated eyelids showed significant muscle loss compared with the control eyelids. Higher doses of doxorubicin alone (*) showed a significant increase in muscle loss compared with the cyclosporin and doxorubicin co-treated eyelids. There was no significant difference between muscle loss in any of the eyelids treated with cyclosporin and doxorubicin.

to exert its effects. Cyclosporin helps maintain cellular calcium homeostasis in the presence of free-oxygen radicals, and this may play a role in its protective action on the skin. Cyclosporin binds to a cytosolic protein named cyclophilin. Cyclophilin is found in most tissues of the body, including skin. The effect of cyclosporin on epithelial cell turnover is unclear; there are reports of inhibitory and noninhibitory effects. Cyclosporin has been used topically and systemically to treat inflammatory skin disorders with good success. The mechanism for the ability of cyclosporin to ameliorate the acute inflammatory reaction caused by doxorubicin is unknown.

Cyclosporin has been shown to increase the toxicity of doxorubicin when this combination of drugs is used for cancer treatment, and it increases the translocation of doxorubicin to the nucleus, thus increasing its pharmacologic effect. It binds to P-glycoprotein, increasing the toxicity of doxorubicin in multidrug resistant cells, mainly by decreasing efflux of doxorubicin from treated cells. Cyclosporin significantly increases the amount of doxorubicin present when used in conjunction with doxorubicin treatment. Cyclosporin inhibits the cytochrome P-450 enzyme system, the pathway involved in the breakdown of doxorubicin, to its inactive aglycone prod-

FIGURE 6. Doxorubicin injections into the eyelid resulted in a significant increase in vascular permeability and inflammation (*). Both 1 mg and 2 mg cyclosporin injections 20 minutes before doxorubicin injection reduced this inflammatory effect, but there was still increased inflammation compared with controls (*). The 2-mg dose of cyclosporin reduced the inflammatory effect of doxorubicin to a statistically significant level (**) compared to doxorubicin alone.
Doxorubicinol is a major active metabolite of doxorubicin. It is less acutely myotoxic than doxorubicin, and this may explain the slight muscle-protective effect of these cotreatments. One of the proposed mechanisms for the muscle-toxic properties of doxorubicin is its formation of oxygen radicals. Cyclosporin has been shown to be a potent inhibitor of prooxidant-induced calcium release from mitochondria, which in turn protects against mitochondrial injury. This may play a role in the muscle-protective effect of cyclosporin when injected before doxorubicin in rabbit eyelids. Injection of cyclosporin into the eyelids before doxorubicin treatment substantially delays the onset, decreases the duration, and could prevent the development of eyelid skin injury. Cyclosporin pretreatment did not increase muscle loss but, rather, had a slight myoprotective effect compared with doxorubicin only. Because of its skin-protective effects, this combination of cyclosporin injection followed by doxorubicin injection has the potential to improve patient acceptance of doxorubicin chemomyectomy for the treatment of blepharospasm and hemifacial spasm.

Further studies are required to determine if immediate or systemic cyclosporin posttreatment could be useful in preventing the skin necrosis that may follow inadvertent infiltration of tissue at the site of the intravenous administration of doxorubicin during cancer chemotherapy. It should be noted that sites chosen for intravenous administration are fundamentally different because the skin does not overlie subcutaneous muscle as is the case in the eyelid.

Key Words
chemomyectomy, facial muscles, blepharospasm, hemifacial spasm, myostatin, orbicularis oculi muscle

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